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(54) Title: p19: A CELL CYCLE INHIBITOR

(57) Abstract

The cell cycle inhibitor p19 is described, including polypeptide and nucleic acid sequences, and cells related thereto. Antibodies to p19 are also presented.

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p19: A CELL CYCLE INHIBITOR

This invention was made with Government support under Grant (Contract) No. MCB-940576 awarded by the National Science Foundation. The Government has certain rights in this invention.

This invention relates to novel polypeptides, nucleic acid sequences and antibodies to the polypeptides of, among other things, a cell cycle inhibitor.

BACKGROUND OF THE INVENTION

Apoptosis in immature T cells and T cell hybridomas, which may relate to negative selection during T cell development, can be initiated by signals through the T-cell receptor/CD3 complex (Mercep *et al.* (1989) *J. Immunol.* 142: 4085-4092; Shi *et al.* (1990) *J. Immunol.* 144: 3326-3333; Smith *et al.* (1989) *Nature* 337: 181-184). This process of activation-induced apoptosis (anti-CD3 apoptosis) consists of two distinct phases. The first phase is a cell cycle block at the G1/S transition, followed by a second phase with generation of apoptotic DNA ladders (Mercep *et al.* (1989) *J. Immunol.* 142: 4085-4092). The second phase requires extracellular calcium and can be inhibited by the immunosuppressive drug cyclosporin A (Mercep *et al.* (1989) *J. Immunol.* 142: 4085-4092). Nur77 (NGFI-B), an orphan steroid receptor, is induced during anti-CD3 apoptosis by calcium signaling events, and it plays an essential role in the cell death process (Liu *et al.* (1994) *Nature* 367: 281-284, Woronicz *et al.* (1994) *Nature* 367: 277-281). Dominant negative Nur77 can block apoptosis but not the IL-2 production of anti-CD3 treated T cell hybridomas (Woronicz *et al.* (1994) *Nature* 367: 277-281). Thus, Nur77 is involved in the second phase of anti-CD3 T cell apoptosis.

Anti-CD3 death in T cell hybridomas is accompanied by a G1 cell cycle block. In all organisms studied so far, cell cycle progression is mediated by cyclin-dependent kinases (CDKs) that consist of a catalytic subunit (CDK) and a regulatory subunit (cyclin). In mammalian cells, cyclinE/CDK2 and cyclinD/CDK4 or cyclinD/CDK6, which are active in the G1 phase, control the

G1 to S transition (Nurse (1990) *Nature* 344: 503-508; Pines (1993) *Trends Biochem. Sci.* 18: 195-197; Reed *et al.* (1992) *Ann. Rev. Cell. Biol.* 8: 529-561; Sherr (1993) *Cell* 73: 1059-1065 and references therein). There are at least three different D type cyclins, with T cells expressing cyclin D2 and D3 and two cyclin D associating kinases, CDK4 and CDK6. One of their substrates is the retinoblastoma (Rb) protein which upon phosphorylation releases the E2F transcription factor. E2F in turn activates genes that are required for the S phase (Sherr (1993) *Cell* 73: 1059-1065). Cell cycle control in G2/M and S phase is mediated by a different set of cyclins and CDKs. These are cyclin B/CDC2 and cyclinA/CDK2, which are active in G2/M and S phase, respectively. Their activities are required for cell entry into mitosis (Nurse (1990) *Nature* 344: 503-508; Pines (1993) *Trends Biochem. Sci.* 18: 195-197; Reed *et al.* (1992) *Ann. Rev. Cell. Biol.* 8: 529-561; Sherr (1993) *Cell* 73: 1059-1065).

Activity of cyclin/CDK kinases is subjected to several levels of regulation, including the action of cell cycle inhibitors. Several of these inhibitors were recently isolated. p21 (also known as Waf1, Cip1, Sdi1 or CAP20) is transcriptionally regulated by p53 and by processes leading to senescence (Eldeiry *et al.* (1993) *Cell* 75: 817-825; Gu *et al.* (1993) *Nature* 366: 707-710, Harper *et al.* (1993) *Cell* 75: 805-816; Noda *et al.* (1994) *Exp. Cell Res.* 211: 90-98, Xiong *et al.* (1993) *Nature* 366: 701-704). It associates with G1 cyclins as well as mitotic cyclins and plays an important role in the assembly of the cyclin/CDK complexes and in DNA replication (Li *et al.* (1994) *Nature* 371: 534-537; Zhang *et al.* (1994) *Genes Dev.* 8: 1750-1758). A closely related protein, p27 is implicated in the G1 phase arrest by TGF-β, cAMP and cell-cell contact (Kato *et al.* (1994) *Cell* 79: 487-496; Polyak *et al.* (1994) *Genes Dev.* 8: 9-22; Polyak *et al.* (1994) *Cell* 78: 59-66, Toyoshima and Hunter (1994) *Cell* 78: 67-74). It also associates with a variety of cyclin/CDK kinases.

In contrast, a different group of cell cycle inhibitors, p16^{ink4} and p15^{ink4B} only associate with the G1 cyclin-dependent kinases CDK4 and CDK6. Their inhibitory activity is restricted to the cyclinD/CDK4 and cyclinD/CDK6 kinases (Hannon *et al.* (1994) *Nature* 371: 257-260; Serrano

et al. (1993) *Nature* 366: 704-707). The predicted amino acid sequence of p16 and p15 contains four ankyrin repeats (Hannon *et al.* (1994) *Nature* 371: 257-260; Serrano *et al.* (1993) *Nature* 366: 704-707). The p16^{ink4} and p15^{ink4b} genes are homologous, with 44% identity in the first 50 amino acids and 97% identity in the last 3 ankyrin domains (Hannon *et al.* (1994) *Nature* 371: 257-260; Serrano *et al.* (1993) *Nature* 366: 704-707). The genes encoding p16 and p15 are located on human chromosome 9p21, a site of frequent chromosomal deletions in many human tumor cell types (Kamb *et al.* (1994) *Science* 264: 436-440, Nobori *et al.* (1994) *Nature* 368: 753-756). Mutations in the p16 gene are frequently detected in primary melanoma cells (Hussussian *et al* (1994) *Nature Genetics* 8: 15-21; Kamb *et al.* (1994) *Nature Genetics* 8: 23-26).

SUMMARY OF THE INVENTION

The present invention results from the discovery of two novel cell cycle regulatory proteins, human p19 and mouse p19. The most similar described protein (p16) is less than 50% similar to the newly discovered p19 proteins. Accordingly, the present invention provides novel p19 polypeptides, nucleic acids encoding p19 polypeptides, recombinant cells which include nucleic acids encoding p19 polypeptides, antibodies to the newly discovered p19 polypeptides, and isolated cells expressing antibodies to p19 proteins.

The present invention provides isolated polypeptides comprising at least 10 contiguous amino acids from a polypeptide sequence selected from the group consisting essentially of SEQ ID NO:2 and SEQ ID NO:4, wherein the polypeptide, when presented as an immunogen, elicits the production of an antibody which specifically binds to a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4, and the polypeptide does not bind to antisera raised against a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4 which has been fully immunosorbed with a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4. Such polypeptides include the polypeptides of SEQ ID NO:2 and SEQ ID NO:4. Particularly desired polypeptides inhibit the kinase activity of cyclin

D1/CDK4, and/or cyclinD/CDK6, bind to CDK4 and CDK6 *in vitro* and *in vivo* and do not inhibit the kinase activity of cyclin E/CDK2. Full-length polypeptides are typically about 19 kDa in size, although they are larger when incorporated into a construct such as an immunological vector. The polypeptides of the present invention are present in several forms, including isolated naturally occurring polypeptides, recombinantly produced polypeptides, and as portions of immunological or expression vectors such as fusion proteins.

The present invention also provides isolated nucleic acids which encode the polypeptides described above. Exemplary nucleic acids include those described in SEQ ID NO:1 and SEQ ID NO:3. In preferred embodiments, the nucleic acid is part of a recombinant vector such as a plasmid or virus. In particularly preferred embodiments, the nucleic acid is incorporated into an expression vector for the production of the polypeptides of the present invention. In preferred embodiments, the nucleic acid selectively hybridizes to either the nucleic acid of SEQ ID NO:1, or SEQ ID NO:3, in the presence of competitive DNA such as a human or mouse genomic library, under stringent hybridization conditions. For instance, in preferred embodiments, the nucleic acid encoding a murine p19 protein selectively hybridizes to the nucleotide sequence of SEQ ID NO:3, present either as a component of a murine genomic library, or added to a murine genomic library (*e.g.,* as cloned DNA) under hybridization conditions of 42°C and 50% formamide and stays detectably bound to the nucleic acid of SEQ ID NO:3 under wash conditions of 2xSSC and 0.1% SDS at 65°C for at least 20 minutes. The nucleic acid sequence may encode, *e.g.,* a murine p19 polypeptide with complete sequence identity to a naturally occurring murine p19 protein. The nucleic acid may also encode a murine polypeptide which is not identical to a naturally occurring p19 polypeptide, such as a fusion protein, or a genetically engineered mutant p19 protein which retains the bases critical for p19 function or immunogenicity as described herein. Similarly, in other preferred embodiments, the nucleic acid encoding a human p19 polypeptide selectively hybridizes to the nucleotide sequence of SEQ ID NO:1, present either as a component of a human genomic library, or added to a human genomic library (*e.g.,* as cloned DNA) under

hybridization conditions of 42°C and 50% formamide and stays detectably bound to the nucleic acid of SEQ ID NO:1 under wash conditions of 2xSSC and 0.1% SDS at 65°C for at least 20 minutes. The nucleic acid sequence may encode, *e.g.*, a human p19 polypeptide with complete sequence identity to a naturally occurring human p19 protein. The nucleic acid may also encode a human polypeptide which is not identical to a naturally occurring p19 polypeptide, such as a fusion protein, or a genetically engineered mutant p19 protein which retains the bases critical for p19 function or immunogenicity as described herein.

Recombinant cells which comprise a nucleic acid of the present invention are also provided, including eukaryotic and prokaryotic cells.

The present invention also provides antibodies in either polyclonal or monoclonal form which bind specifically to the polypeptides of the present invention.

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide

chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies exist *e.g.,* as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)'_2$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The $F(ab)'_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the $F(ab)'_2$ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see, Fundamental Immunology*, Third Edition, W.E. Paul, ed., Raven Press, N.Y. (1993), which is incorporated herein by reference, for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies.

The term "immunoassay" is an assay that utilizes an antibody to specifically bind an analyte. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the analyte.

The terms "isolated" "purified" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state.

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides.

The term "nucleic acid probe" refers to a molecule which binds to a specific sequence or subsequence of a nucleic acid. A probe is preferably a nucleic acid which binds through complementary base pairing to the full sequence or to a subsequence of a target nucleic acid. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labelled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labelled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

A "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P, fluorescent dyes, electron-dense reagents, enzymes (*e.g.,* as commonly used in an ELISA), biotin, dioxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available (*e.g.,* the peptide of SEQ ID NO:2 can be made detectible, *e.g.,* by incorporating a radio-label into the peptide, and used to detect antibodies specifically reactive with the peptide).

A "labeled nucleic acid probe" is a nucleic acid probe that is bound, either covalently, through a linker, or through ionic, van der Waals or hydrogen bonds to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe.

The term "recombinant" when used with reference to a cell indicates that the cell encodes a DNA whose origin is exogenous to the cell-type. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell.

The term "identical" in the context of two nucleic acids or polypeptide sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence. Optimal alignment of sequences for comparison can be conducted, *e.g.,* by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

The term "substantial identity" or "substantial similarity" in the context of a polypeptide indicates that a polypeptides comprises a sequence with at least 70% sequence identity to a reference sequence, or preferably 80%, or more preferably 85% sequence identity to the reference sequence, or most preferably 90% identity over a comparison window of about 10-20 amino acid residues. An indication that two polypeptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution.

An indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence dependent and are different under different environmental parameters. Generally, stringent conditions are selected to be about 5° C to 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50%

of the target sequence hybridizes to a perfectly matched probe. However, nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.,* when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

The phrases "specifically binds to a protein" or "specifically hybridizes to" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind preferentially to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions requires an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a sequence alignment between human p19 protein (SEQ ID NO:2, mouse p19 protein (SEQ ID NO:4), human p16 protein (SEQ ID NO:5) and human p15 protein (SEQ ID NO:6).

DETAILED DESCRIPTION OF THE INVENTION

As described above, the invention relates to the discovery of human and mouse derived p19, new cell cycle regulatory proteins. Thus, polypeptides derived from p19, nucleic acid encoding such polypeptides and antibodies to the polypeptides are described here as well as uses for such

compositions, all described below. Most interestingly, p19 mediates apoptosis and cell cycle regulation.

Apoptosis and the cell cycle are two fundamental processes in biology. Cell cycle regulation is relatively well characterized, whereas the molecular mechanism(s) of apoptosis is not as clear, partly because different inducing agents may cause cell death through distinct cellular proteins. In radiation induced apoptosis, for example, cell death is mediated by p53, which transcriptionally regulates the level of the cell cycle inhibitor p21 (Eldeiry *et al.* (1993) *Cell* 75: 817-825; Harper *et al.* (1993) *Cell* 75: 805-816). The relationship of p21 to the apoptotic function of p53 is not entirely clear, although radiation induced apoptosis is usually accompanied by a G1 arrest (Caelles *et al.* (1994) *Nature* 370: 220-223).

In activation induced apoptosis of T cell hybridomas, which may mimic the process of negative selection in T cell development, induction of the Nur77 orphan steroid receptor is required (Liu *et al.* (1994) *Nature* 367: 281-284, Woronicz *et al.* (1994) *Nature* 367: 277-281). Interestingly, when T cell hybridomas are induced to die through the T-cell receptor signals, they are also arrested at the G1 state of the cell cycle. p19 is a novel CDK4/CDK6 inhibitor which plays a role in the regulation of the cell cycle, *e.g.,* in T cells.

The G1/S checkpoint is mainly dictated by the kinase activity of the cyclinD/CDK4, cyclinD/CDK6, and cyclinE/CDK2 complex. These G1 kinases are regulated by cell cycle inhibitors, which arrest the cells at the G1 growth phase. In T cell hybridomas, addition of anti-T-cell receptor antibody results in G1 arrest and also apoptosis. p19 associates with CDK4 but not with CDK2, CDC2 or any of the cyclins (*i.e.,* A,B,D1,D2,D3). p19 protein inhibits the kinase activity of cyclinD1/CDK4 but not that of cyclinE/CDK2. Furthermore, we found that p19 associates with both CDK4 and CDK6 *in vivo* in the T cell hybridoma DO11.10.

In searching for protein(s) that interact with Nur77 using yeast two hybrid screening, we isolated novel homologous mouse and human cell cycle inhibitors, which we designated mouse p19 and human p19. Sequencing of the human and mouse cDNA revealed 164 and 165 amino acid open reading

frames, respectively. The human p19 gene maps to chromosome 19p13, distinct from that of other known cell cycle inhibitors such as p18, p16 and p15. The proteins share extensive homology with the well-known cell cycle inhibitor p16 (*see*, Figure 1). The deduced p19 amino acid sequence has 48% sequence identity with p16, and has four ankyrin repeats. Its mRNA is expressed in all cell types examined. A p19 fusion protein (*See*, Examples) associates *in vitro* with CDK4 but not with CDK2, CDC2 or cyclin A, B, E, or D1-D3.

Addition of p19 protein inhibits the *in vitro* kinase activity of cyclinD/CDK4 but not that of cyclinE/CDK2. In T cell hybridoma DO11.10, p19 associates with CDK4 and CDK6. p19 protein is similar to p16 and has all the properties of a cell cycle inhibitor. This is consistent with the ability of p19 to associate with the G1 specific kinases, CDK4 and CDK6, but not with other cell cycle kinases or cyclins.

We also found that p19 associates with CDK4 and CDK6 *in vivo*, although its expression does not change appreciably when T cell hybridomas are arrested at the G1 phase through T cell receptor signals. This is similar to p16, for which regulation is still poorly understood. In contrast, p15 protein level in keratinocytes increases dramatically in response to TGF-β, leading to G1 arrest. Thus, p19 may participate in the G1 arrest of T cells through post-translational modification.

In cloning the p19 cDNA through a yeast two hybrid screening strategy with the Nur77 DNA binding domain as a bait, we found that protein-protein interaction between p19 and Nur77 was relatively strong. The interaction survived numerous tests used for the screening process. *In vitro* translated full length Nur77 protein, however, does not associate with the GST-p19 fusion protein. Whether Nur77 associates with p19 *in vivo* has not been tested. Clear differences, however, are evident between *in vitro* translated and *in vivo* Nur77 protein, including the presence of foreign GST polypeptide sequences in the fusion protein, and native *in vivo* phosphorylation.

An analogous situation exists in yeast, where a weak association between the transcription factor Pho4 and the cell cycle inhibitor Pho81 is seen

using the yeast two hybrid strategy, but not by co-immunoprecipitation using the corresponding antibodies (Hirst *et al.* (1994) *EMBO J.* 13: 5410-5420; Schneider *et al.* (1994) *Science* 266: 122-125). Pho81 contains four ankyrin repeats similar to that of the human p16/p19 cell cycle inhibitor family. Under high phosphate conditions, the yeast Pho81 mediates interaction between the Pho4 transcription factor and Pho80/Pho85 cyclin/CDK complex (Hirst *et al.* (1994) *EMBO J.* 13: 5410-5420; Schneider *et al.* (1994) *Science* 266: 122-125). Phosphorylation of Pho4 by the Pho85 CDK kinase leads to inactivation of the Pho4 DNA binding activity. When phosphate starvation occurs, however, Pho81 inactivates the Pho80/Pho85 complex and dissociates from Pho4 at the same time. The resulting unphosphorylated Pho4 is an active protein (Hirst *et al.* (1994) *EMBO J.* 13: 5410-5420; Schneider *et al.* (1994) *Science* 266: 122-125). Thus, the cell cycle dependent kinases may directly modulate the activity of a transcription factor through interaction with the p16 family of cell cycle inhibitors. For instance, induction of Nur77 mRNA by either serum or NGF or anti-TCR antibodies may initially lead to an active phosphorylated protein. Apoptosis associated activity of Nur77 protein may be inactivated by a second phosphorylation event mediated by the CDK6/cyclin complex (or CDK4/cyclin complex) through interaction with p19. When cells are arrested at G1 in anti-TCR treated cells, p19 may dissociate itself from Nur77, and inactivate the CDK6/cyclin complex. The lack of a second phosphorylation event may result in an active Nur77 protein which is then involved in the apoptotic process of T cell hybridomas. For thymocytes, where most of the cells are at the G0/G1state, expression of Nur77 can lead to apoptosis. This model is based on a known biological system in yeast and provides a molecular link between G1 arrest and apoptosis in T cells.

General Techniques

Cloning, PCR, LCR, TAS, 3SR, And QB Amplification

The present invention is used in conjunction with techniques such as PCR, TAS, 3SR, QB amplification and cloning, to amplify a nucleic acid in a biological sample which encodes a p19 polypeptide.

The presence of p19 nucleic acid in a biological sample is useful, *e.g.,* as a probe to asses *in vivo* and *in situ* RNA expression, or in DNA forensic analysis such as DNA fingerprinting. p19 probes are also useful in florescent karyotyping analysis to monitor the presence of, *e.g.,* human chromosome 19. Because p19 is shown herein to map to human chromosome 19p13, one of skill can use the gene as a probe to asses whether there are any gross chromosomal abnormalities in this region of chromosome 19. This is useful, for instance, in *in utero* screening of a fetus to monitor for the presence of chromosomal abnormalities.

The nucleic acids of the present invention are cloned, or amplified by *in vitro* methods, such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (3SR) and the Qβ replicase amplification system (QB). A wide variety of cloning and *in vitro* amplification methodologies are well-known to persons of skill. Examples of these techniques and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook *et al.*); *Current Protocols in Molecular Biology*, F.M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel); Cashion et al., U.S. patent number 5,017,478; and Carr, European Patent No. 0,246,864. Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods are found in Berger, Sambrook, and Ausubel, as well as Mullis et al., (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis et al. eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3, 81-94; (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86, 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.* (1989) *J. Clin. Chem* 35, 1826; Landegren *et al.*, (1988)

Science 241, 1077-1080; Van Brunt (1990) *Biotechnology* 8, 291-294; Wu and Wallace, (1989) *Gene* 4, 560; and Barringer *et al.* (1990) *Gene* 89, 117.

Antibodies to p19

Antibodies are raised to the polypeptides of the present invention, including individual, allelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms. Additionally, antibodies are raised to these polypeptides in either their native configurations or in non-native configurations. Anti-idiotypic antibodies can also be generated. Many methods of making antibodies are known to persons of skill. The following discussion is presented as a general overview of the techniques available; however, one of skill will recognize that many variations upon the following methods are known.

a. *Antibody Production*

A number of immunogens are used to produce antibodies specifically reactive with p19 (mouse or human) polypeptides. Recombinant or synthetic polypeptides of 10 amino acids in length, or greater, selected from sub-sequences of SEQ ID NO:2 or SEQ ID NO:4 are the preferred polypeptide immunogen for the production of monoclonal or polyclonal antibodies. In one class of preferred embodiments, an immunogenic peptide conjugate is also included as an immunogen. Naturally occurring polypeptides are also used either in pure or impure form.

Recombinant polypeptides are expressed in eukaryotic or prokaryotic cells and purified using standard techniques. The polypeptide, or a synthetic version thereof, is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies can be generated for subsequent use in immunoassays to measure the presence and quantity of the polypeptide.

Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen, preferably a purified polypeptide, a polypeptide coupled to an appropriate carrier (*e.g.,* GST, keyhole limpet

hemanocyanin, *etc.*), or a polypeptide incorporated into an immunization vector such as a recombinant vaccinia virus (*see*, U.S. Patent No. 4,722,848) is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired. *See, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY, which are incorporated herein by reference, and the examples below.

Antibodies, including binding fragments and single chain recombinant versions thereof, against predetermined fragments of p19 polypeptides are raised by immunizing animals, *e.g.*, with conjugates of the fragments with carrier proteins as described above. Typically, the immunogen of interest is a peptide of at least about 3 amino acids, more typically the peptide is 5 amino acids in length, preferably, the fragment is 10 amino acids in length and more preferably the fragment is 15 amino acids in length or greater. The peptides are typically coupled to a carrier protein (*e.g.*, as a fusion protein), or are recombinantly expressed in an immunization vector. Antigenic determinants on peptides to which antibodies bind are typically 3 to 10 amino acids in length.

Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies are screened for binding to normal or modified polypeptides, or screened for agonistic or antagonistic activity, *e.g.*, activity mediated through p19. Specific monoclonal and polyclonal antibodies will usually bind with a K_D of at least about .1 mM, more usually at least about 50 μM, and most preferably at least about 1 μM or better.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of techniques for preparing such monoclonal antibodies are found in,

e.g., Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, *Supra*; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497. Summarized briefly, this method proceeds by injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate (prferably mammalian) host. The polypeptides and antibodies of the present invention are used with or without modification, and include chimeric antibodies such as humanized murine antibodies.

Other suitable techniques involve selection of libraries of recombinant antibodies in phage or similar vectors. See, Huse *et al.* (1989) *Science* 246: 1275-1281; and Ward, *et al.* (1989) *Nature* 341: 544-546.

Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos.

3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced. See, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al.* (1989) *Proc. Nat'l Acad. Sci. USA* 86: 10029-10033.

The antibodies of this invention are also used for affinity chromatography in isolating p19 polypeptides. Columns are prepared, *e.g.,* with the antibodies linked to a solid support, *e.g.*, particles, such as agarose, Sephadex, or the like, where a cell lysate is passed through the column, washed, and treated with increasing concentrations of a mild denaturant, whereby purified p19 polypeptides are released.

The antibodies can be used to screen expression libraries for particular expression products such as mammalian p19. Usually the antibodies in such a procedure are labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

Antibodies raised against p19 polypeptides can also be used to raise anti-idiotypic antibodies. These are useful for detecting or diagnosing various pathological conditions related to the presence of the respective antigens.

b. *Immunoassays*

A particular protein can be quantified by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see Stites and Terr (eds.) 1991 *Basic and Clinical Immunology* (7th ed.). Moreover, the immunoassays of the present invention can be performed in any of several configurations, *e.g.,* those reviewed in Maggio (ed.) (1980) *Enzyme Immunoassay* CRC Press, Boca Raton, Florida; Tijan (1985) "Practice and Theory of Enzyme Immunoassays," *Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers B.V., Amsterdam; Harlow and Lane, *supra*; Chan (ed.) (1987) *Immunoassay: A Practical Guide* Academic Press, Orlando, FL; Price and Newman (eds.) (1991) *Principles and Practice of Immunoassays* Stockton Press, NY; and Ngo (ed.) (1988) *Non-isotopic Immunoassays* Plenum Press, NY.

Immunoassays also often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte. The labeling agent may itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled p19 peptide or a labeled anti-p19 antibody. Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody/p19 complex, or to a modified capture group (*e.g.*, biotin) which is covalently linked to the p19 peptide or anti-p19 antibody.

In a preferred embodiment, the labeling agent is an antibody that specifically binds to the capture agent (anti-p19). Such agents are well known to those of skill in the art, and most typically comprise labeled antibodies that specifically bind antibodies of the particular animal species from which the capture agent is derived (*e.g.*, an anti-idiotypic antibody). Thus, for example, where the capture agent is a mouse derived anti-human p19 antibody, the label agent may be a goat anti-mouse IgG, *i.e.*, an antibody specific to the constant region of the mouse antibody.

Other proteins capable of specifically binding immunoglobulin constant regions, such as streptococcal protein A or protein G are also used as the labeling agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species. See, generally Kronval, *et al.*, (1973) *J. Immunol.*, 111:1401-1406, and Akerstrom, *et al.*, (1985) *J. Immunol.*, 135:2589-2542.

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays are carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 5°C to 45°C.

(i) Non-Competitive Assay Formats

Immunoassays for detecting p19 may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case p19) is directly measured. In one preferred "sandwich" assay, for example, the capture agent (*e.g.,* anti-p19 antibodies) are bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture p19 present in the test sample. The p19 thus immobilized is then bound by a labeling agent, such as a second human p19 antibody bearing a label. Alternatively, the second p19 antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived.

Sandwich assays for p19 may be constructed. As described above, the immobilized anti-p19 specifically binds to p19 present in the sample. The labeled anti-p19 then binds to the already bound p19. Free labeled anti-p19 is washed away and the remaining bound labeled anti-p19 is detected (*e.g.,* using a gamma detector where the label is radioactive).

(ii) Competitive Assay Formats

In competitive assays, the amount of analyte (*e.g.,* p19) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte displaced (or competed away) from a capture agent (*e.g.,* anti p19 antibody) by the analyte present in the sample. In one competitive assay, a known amount of analyte is added to the sample and the sample is contacted with a capture agent, in this case an antibody that specifically binds the analyte. The amount of analyte bound to the antibody is inversely proportional to the concentration of analyte present in the sample.

In a particularly preferred embodiment, the capture agent is immobilized on a solid substrate. The amount of p19 bound to the capture agent is determined either by measuring the amount of p19 present in an p19/antibody complex, or alternatively by measuring the amount of remaining uncomplexed p19. The amount of p19 may be detected by providing a labeled p19.

A hapten inhibition assay is another preferred competitive assay. In this assay, a known analyte, in this case p19, is immobilized on a solid substrate. A known amount of anti-p19 antibody is added to the sample, and the sample is then contacted with the immobilized p19. In this case, the amount of anti-p19 antibody bound to the immobilized p19 is proportional to the amount of p19 present in the sample. Again the amount of immobilized antibody iis detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled, or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

(iii) Generation of pooled antisera for use in immunoassays.

A p19 protein that specifically binds to or that is specifically immunoreactive with an antibody generated against a defined immunogen, such as an immunogen consisting of the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4, is determined in an immunoassay. The immunoassay uses a polyclonal antiserum which was raised either to the protein of SEQ ID NO:2 or SEQ ID NO:4 (the immunogenic polypeptide). This antiserum is selected to have low crossreactivity against other cell cycle inhibitors and any such crossreactivity is removed by immunoabsorbtion prior to use in the immunoassay (*e.g.,* by immunosorbtion of the antisera related cell-cycle inhibitors such as p15 and p16).

In order to produce antisera for use in an immunoassay, the polypeptide of SEQ ID NO:2 or SEQ ID NO:4 is isolated as described herein. For example, recombinant protein can be produced in a mammalian or other eukaryotic cell line. An inbred strain of mice is immunized with the protein of SEQ ID NO:2 or SEQ ID NO:4 using a standard adjuvant, such as Freund's adjuvant, and a standard mouse immunization protocol (see Harlow and Lane, *supra*). Alternatively, a synthetic polypeptide derived from the sequences disclosed herein and conjugated to a carrier protein is used as an immunogen. Polyclonal sera are collected and titered against the immunogenic polypeptide in an immunoassay, for example, a solid phase immunoassay with the immunogen

immobilized on a solid support. Polyclonal antisera with a titer of 10^4 or greater are selected and tested for their cross reactivity against cell cycle inhibitors, using a competitive binding immunoassay such as the one described in Harlow and Lane, *supra*, at pages 570-573. Preferably two cell cycle inhibitors (*e.g.*, p15 and p16) are used in this determination in conjunction with either human or mouse p19 (depending on which p19 was used as the immunogen). In conjunction with mouse p19, or human p19, the cell cycle inhibitors p15 or p16 are used as competitors to identify antibodies which are specifically bound by a p19. The competitive cell cycle inhibitors can be produced as recombinant proteins and isolated using standard molecular biology and protein chemistry techniques as described herein.

Immunoassays in the competitive binding format are used for crossreactivity determinations. For example, the immunogenic polypeptide is immobilized to a solid support. Proteins added to the assay compete with the binding of the antisera to the immobilized antigen. The ability of the above proteins to compete with the binding of the antisera to the immobilized protein is compared to the immunogenic polypeptide. The percent crossreactivity for the above proteins is calculated, using standard calculations. Those antisera with less than 10% crossreactivity with p 15 and p16 are selected and pooled. The cross-reacting antibodies are then removed from the pooled antisera by immunoabsorbtion with the p15 and p16.

The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay as described herein to compare a second "target" polypeptide to the immunogenic polypeptide. In order to make this comparison, the two polypeptides are each assayed at a wide range of concentrations and the amount of each polypeptide required to inhibit 50% of the binding of the antisera to the immobilized protein is determined using standard techniques. If the amount of the target polypeptide required is less than twice the amount of the immunogenic polypeptide that is required, then the target polypeptide is said to specifically bind to an antibody generated to the immunogenic protein. As a final determination of specificity, the pooled antisera is fully immunosorbed with the immunogenic polypeptide until no

binding to the polypeptide used in the immunosorbtion is detectable. The fully immunosorbed antisera is then tested for reactivity with the test polypeptide. If no reactivity is observed, then the test polypeptide is specifically bound by the antisera elicited by the immunogenic protein.

Assays for p19

A. *Sample Collection and Processing*

p19 is preferably quantified in a biological sample, such as a cell, or a tissue sample derived from a patient. In a preferred embodiment, p19 is quantified in T cells derived from whole blood or blood derivatives such as blood serum. Blood samples are isolated from a patient according to standard methods well known to those of skill in the art, most typically by venipuncture. Although the sample is typically taken from a human patient, the assays can be used to detect p19 in samples from mammals in general, such as dogs, cats, sheep, cattle and pigs, and most particularly primates such as chimpanzees, gorillas, macaques, and baboons and rodents such as mice, rats, squirrels, and guinea pigs. In this regard, one of skill will recognize that the homologous human and murine p19 proteins are over 80% similar, despite the substantial evolutionary divergence of humans and mice. One of skill would expect that the human p19 gene can be used to isolate p19 genes from any species more closely related to humans than mice, and that the murine p19 gene can be used to isolate p19 genes from species more closely related to mice than to humans, using standard techniques. Such standard techniques are well known to persons of skill, and include *e.g.,* screening a genomic or cDNA library with the desired p19 sequence, screening an expression library with antibodies to the desired p19 polypeptide, and performing PCR using appropriate primers from the desired p19 sequence. As described below these standard techniques were sufficient to isolate human p19 using the mouse p19 as a probe. Given the substantial sequence conservation of the gene, one of skill would expect to be able to isolate the p19 sequence from a wide array of species using standard techniques.

The sample may be pretreated as necessary by dilution in an appropriate buffer solution or concentrated, if desired. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris, or the like, at physiological pH can be used.

B. *Quantification of p19 peptides.*

p19 peptides may be detected and quantified by any of a number of means well known to those of skill in the art. These include analytic biochemical methods such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, and various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like.

C. *Reduction of Non-Specific Binding*

One of skill will appreciate that it is often desirable to reduce non-specific binding in immunoassays and during analyte purification. Where the assay involves an antigen, antibody, or other capture agent immobilized on a solid substrate, it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific binding are well known to those of skill in the art. Typically, this involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used.

D. *Other Assay Formats*

Western blot analysis can also be used to detect and quantify the presence of p19 in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind p19. The anti-p19 antibodies

specifically bind to p19 on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (*e.g.,* labeled sheep anti-mouse antibodies where the antibody to p19 is a murine antibody) that specifically bind to the anti-p19.

Other assay formats include liposome immunoassays (LIAs), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (see, Monroe *et al., (1986) Amer. Clin. Prod. Rev.* 5:34-41), which is incorporated herein by reference.

E. *Labels*

The labeling agent can be, *e.g.*, a monoclonal antibody, a polyclonal antibody, a p19 binding protein or complex such as those described herein, or a polymer such as an affinity matrix, carbohydrate or lipid. Detection may proceed by any known method, such as immunoblotting, western analysis, gel-mobility shift assays, fluorescent *in situ* hybridization analysis (FISH), tracking of radioactive or bioluminescent markers, nuclear magnetic resonance, electron paramagnetic resonance, stopped-flow spectroscopy, column chromatography, capillary electrophoresis, or other methods which track a molecule based upon an alteration in size and/or charge. The particular label or detectable group used in the assay is not a critical aspect of the invention. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads^{TM}), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads.

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on the sensitivity required, ease of conjugation of the compound, stability requirements, available instrumentation, and disposal provisions.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (*e.g.*, streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various labelling or signal producing systems which may be used, see, U.S. Patent No. 4,391,904, which is incorporated herein by reference.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence, *e.g.*, by microscopy, visual inspection, via photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing appropriate substrates for the enzyme and detecting

the resulting reaction product. Finally, simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

F. *Substrates*

As mentioned above, depending upon the assay, various components, including the antigen, target antibody, or anti-human antibody, may be bound to a solid surface. Many methods for immobilizing biomolecules to a variety of solid surfaces are known in the art. For instance, the solid surface may be a membrane (*e.g.*, nitrocellulose), a microtiter dish (*e.g.*, PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dipstick (*e.g.* glass, PVC, polypropylene, polystyrene, latex, and the like), a microcentrifuge tube, or a glass, silica, plastic, metallic or polymer bead. The desired component may be covalently bound, or noncovalently attached through nonspecific bonding.

A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and the like. Other materials which may be employed, include paper, glasses, ceramics, metals, metalloids, semiconductive materials, cements or the like. In addition, substances that form gels, such as proteins (*e.g.*, gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides can be used. Polymers which form

several aqueous phases, such as dextrans, polyalkylene glycols or surfactants, such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium salts and the like are also suitable. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

In preparing the surface, a plurality of different materials may be employed, *e.g.,* as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be used to avoid non-specific binding, simplify covalent conjugation, enhance signal detection or the like.

If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature. See, for example, *Immobilized Enzymes*, Ichiro Chibata, Halsted Press, New York, 1978, and Cuatrecasas, *J. Biol. Chem.* 245 3059 (1970) which are incorporated herein by reference.

In addition to covalent bonding, various methods for noncovalently binding an assay component can be used. Noncovalent binding is typically nonspecific absorption of a compound to the surface. Typically, the surface is blocked with a second compound to prevent nonspecific binding of labeled assay components. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For example, a surface bearing a lectin such as Concanavalin A will bind a carbohydrate containing compound but not a labeled protein that lacks glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082, which are incorporated herein by reference.

Uses for p19

p19 nucleic acids, *e.g.*, human or mouse p19 DNA or RNA are useful as a component in a forensic assay. For instance, the nucleotide

sequences provided may be labeled using, *e.g.*, ^{32}P or biotin and used to probe standard restriction fragment polymorphism blots, providing a measurable character to aid in distinguishing between individuals. Such probes are used in well-known forensic techniques such as genetic fingerprinting, or related techniques based upon PCR. In addition, nucleotide probes made from p19 sequences are used in *in situ* assays to detect chromosomal abnormalities. For instance, rearrangements in human chromosome 19 are detected via well-known *in situ* techniques such as FISH, using p19 probes in conjunction with other known chromosome 19 markers.

As noted above, the G1/S checkpoint is dictated by the kinase activity of cyclinD/CDK4 and cyclin D/CDK6 complexes. By inhibiting the action of these complexes, p19 peptides may be used to synchronize cell culture systems *in vitro*. This is useful whenever a synchronized cell culture is desired, such as when a metaphase spread of the cells is prepared for chromosomal analysis. In preferred embodiments, fragments of the full-length p19 proteins are used in order to facilitate entry of the peptides into the cell, rather than the full length p19 proteins.

Particularly effective peptides are found using simple screening techniques. In general, sequential fragments of the full-length proteins which include 10-50 amino acids are created, *e.g.,* by deletion and expression of the recombinant gene, or by chemical synthesis of either the relevant p19 nucleic acid fragment (and subsequent subcloning and expression) or chemical synthesis of the desired amino acid polymer using standard automated protein synthetic techniques. The fragments are then used in the assays described herein to determine their ability to block kinase activity of CDK4 and CDK6 complexes. Those fragments which block CDK4/CDK6 kinase activity are used as cell-cycle inhibitors, permitting synchronization of cell cultures.

These peptides are also used as therapeutics to inhibit cell growth *in vivo*, particularly in T cells. Many pathologies are associated with improper T cell regulation including various forms of cancer and immunological disorders.

Antibodies and other binding agents directed towards p19 proteins or nucleic acids may be used to purify the corresponding p19 molecule.

Antibodies and other binding agents are also used in a diagnostic fashion to determine whether p19 components are present in a tissue sample or cell population using well-known techniques described herein. The ability to attach a binding agent to a p19 protein provides a means to diagnose disorders associated with p19 misregulation.

Administration of p19 polypeptides to Patients

The polypeptides of the present invention are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. One skilled in the art will appreciate that suitable methods of administering such compounds in the context of the present invention to a patient are available, and, although more than one route can be used to administer a particular compound, a particular route can often provide a more immediate and more effective reaction than another route. It should be recognized that the administration of peptides are well-known for a variety of diseases, and one of skill is able to extrapolate the information available for use of peptides to treat these other diseases to p19 peptides.

Pharmaceutically acceptable carriers are also well known to those who are skilled in the art. The optimal choice of carrier will be determined in part by the particular compound, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of the pharmaceutical compositions of the present invention.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the compound dissolved in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, tragacanth, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening

agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, such as carriers as are known in the art.

The active ingredient, alone or in combination with other suitable components, can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Suitable formulations for rectal administration include, for example, suppositories, which consist of the active ingredient with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the active ingredient with a base, such as, for example, liquid triglyercides, polyethylene glycols, and paraffin hydrocarbons.

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the

animal over time. The dose will be determined by the strength of the particular compound employed and the condition of the animal, as well as the body weight or surface area of the animal to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular compound in a particular patient. In determining the effective amount of the active ingredient to be administered in the treatment, the physician evaluates circulating plasma levels, peptide toxicities, and conditions associated with improper cell regulation such as tumor growth inhibition, and/or cancer progression.

In the practice of this invention, the compounds can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally for treatment of cancers such as lymphomas, leukemias, and solid tumors. The preferred method of administration of p19 polypeptides will often be oral, rectal or intravenous, but the compounds can be applied in a suitable vehicle for the local and topical treatment of surface cell growth abnormalities such as skin cancers.

These compounds supplement treatment of cell-growth related disorders such as cancer by any appropriate conventional therapy, including cytotoxic agents and biologic response modifiers. The most preferred effective dose *in vivo* will achieve an intra cellular concentration sufficient to block CDK4/CDK6 kinase activity in the region of the cellular disorder. This can be tested empirically using the kinase inhibition tests described herein. The dosage can be administered via single or divided doses.

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially similar results.

EXAMPLES

Example 1: Isolation of p19 using a Yeast two hybrid system.

In searching for protein(s) associating with Nur77 (Christy *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85: 7857-7861; Milbrandt (1988). *Neuron* 1: 183-188), we used a mouse Nur77 cDNA as a bait in a yeast two hybrid screen. To generate a Gal4 fusion construct, a cDNA sequence corresponding to the DNA and ligand binding domains of mouse Nur77 was first amplified by polymerase chain reaction from the N10 plasmid containing Nur77 cDNA (in pKS-bluescript). The primers used were T7 primer at the 3' end and a primer containing the Nur77 sequence starting from nucleotide 869. A BamHI site was introduced at the 5' oligonucleotide. The resulting 1.5kb fragment was cloned into the BamHI site of pSP72 (Promega). A 0.45kb BamHI/AlwNI (filled in) fragment including the DNA binding domain plus the A box was then subcloned into pAS-CYH1 at the BamHI/SalI (filled in) site. This fusion plasmid was used to screen a mouse peripheral blood T cell library in pACT using published procedures (Durfee *et al.* (1993) *Genes Dev.* 7: 555-569; see also Winoto *et al., Molecular and Cellular Biology*, May 1995 issue).

Plasmids encoding fusion proteins of Gal4 DNA binding domain and various Nur77 protein domains were made and tested first for their ability to activate a lacZ reporter gene under the control of several Gal4 DNA binding sites. Neither the Nur77 DNA binding domain (two zinc fingers and A box) nor its C terminal domain contained any transactivation activity. We used the Gal4-Nur77 DNA binding domain construct to screen a mouse peripheral blood T cell cDNA library made in the appropriate plasmid as a fusion protein with the Gal4 activation domain.

Screening was done as described (Durfee *et al.* (1993) *Genes Dev.* 7: 555-569) by first isolating histidine$^+$ colonies and then testing them for expression of the lacZ gene. Clones that showed bait-dependant lacZ expression were chosen for further analysis. Several plasmids encoding proteins that interact with the Nur77 DNA binding domain but not with a series of irrelevant proteins were obtained. Co-transfection of any of these plasmids with the Gal4-Nur77 fusion protein plasmid resulted in activation of the lacZ

gene under Gal4 control. One of these clones contained an open reading frame with homology to the previously published human p16 and p15 cell cycle inhibitors (Hannon *et al.* (1994) *Nature* 371: 257-260; Kamb *et al.* (1994) *Science* 264: 436-440; Serrano *et al.* (1993) *Nature* 366: 704-707), all of which comprise 4 ankyrin repeats (Figure 1). We designated this gene p19 to reflect its protein molecular weight *in vitro* (after transcription and translation) and *in vivo* (detected by immunoprecipitation, *see supra*).

To verify that the newly isolated cDNA was not a mouse homolog of human p16, we screened a human thymus cDNA library with the mouse p19 as a probe. The deduced human and mouse p19 protein sequence showed 81% sequence identity. The human p19 is clearly different from p16, with its deduced sequence sharing 48% sequence identity with human p16 over a stretch of 130 amino acids. The human p19 sequence is also different from the human p15 and p18 sequence (Guan *et al.* (1994) *Genes & Dev.* 8: 2939-2952; Hannon *et al.* (1994) *Nature* 371: 257-260), indicating that p19 is a novel member of the p16 cell cycle inhibitor family.

Northern blot analysis with mRNA from several cell lines showed that p19 mRNA is expressed in all cell types examined. A transcript of approximately 1.4 kb is found in pre-B cell lines (1.8, 22D6, WEHI231), macrophage cells (P388D1), T cells (AO4H5.3, EL4), fibroblast cells (NIH3T3, LTk^-) and erythroleukemia cells (MEL).

Example 2: Chromosome Mapping of p19

In order to see if p19 is associated with any characteristic tumor-specific chromosomal abnormalities, we determined its chromosomal location. Both p16 and p15 genes are located on human chromosome 9p21 (Hannon *et al.* (1994) *Nature* 371: 257-260; Kamb *et al.* (1994) *Science* 264: 436-440; Nobori *et al.* (1994) *Nature* 368: 753-756), a site with frequent deletions in many types of cancerous cells. Mutations at the p16 gene are present in many primary melanoma cells (Hussussian *et al* (1994) *Nature Genetics* 8: 15-21; Kamb *et al.* (1994) *Nature Genetics* 8: 23-26).

A human cosmid library (Stratagene) was screened with human p19 cDNA as a probe. Two overlapping cosmid clones (pCOS3A and pCOS4B) were obtained. They encode the p19 gene as confirmed by Southern blot hybridization and by comparison of the restriction enzyme sites of the putative exons with that of the p19 cDNA. These cosmids were used in a fluorescence *in situ* hybridization to map the chromosomal location of the human p19 gene.

Bromodeoxyuridine-synchronized, phytohemagglutinin-stimulated peripheral blood lymphocytes from a normal donor were used as a source of metaphase chromosomes. Genomic DNA in a cosmid vector was labelled with either digoxigenin-11-UTP or biotin-14-UTP and hybridized overnight at 37°C to fixed metaphase chromosomes according to published methods (Pinkel (1988) *Proc. Natl. Acad. Sci. USA* 85: 9138-9142), except for the inclusion of 33 ug/ml of highly reiterated human DNA self-annealed to Cot1. Signals were detected by incubating the slides with fluorescein-conjugated sheep antidigoxigenin antibodies and Texas-red conjugated avidin followed by counterstaining with 4',6-diamidino-2-phenylindole (DAPI) in antifade. Analysis of 20 hybridized chromosomes were made from digitally acquired merged images that were obtained using a CCD camera and standard software. Fluorescence microscopy was performed with Nikon Optiphot microscope.

Human p19 was found to be located on chromosome 19p13, a region not characteristically involved in tumor-associated chromosomal abnormalities. This assignment was confirmed by using a previously mapped gene DBP, which is located on chromosomal 19q13 as a control. Thus, although p19 is homologous to p16, it is not located on the same chromosome.

Example 3: p19 Associates with CDK4 in vitro, but not with CDK2, CDC2, cyclin A, cyclin B, cyclin D, or cyclin E

To investigate whether p19 associates with components of the cell cycle machinery, we first expressed mouse p19 as a GST fusion protein in *E. coli* and purified it using glutathione agarose beads. For mouse p19, a 1.1 kb XhoI insert from the yeast vector pACT was subcloned into a pSP72 plasmid (Promega) and used for *in vitro* transcription and translation experiments.

Human p19 cDNA clones were isolated from a human thymus lgt11 library (Clontech) using mouse p19 as a probe under low stringency condition. The largest insert (1.3 kb) was then subcloned into the EcoRI site of the pSP72 plasmid (Promega). The mouse p19 was used for all subsequent experiments. For *in vitro* transcription, the CDC2 plasmid was cut with EcoRV and transcribed with SP6 RNA polymerase; the CDK2 plasmid (in pSP72) was cut with HindIII and transcribed with T7 RNA polymerase, the CDK4 plasmid (in pSP72) was cut with HindIII and transcribed with T3 RNA polymerase, the cyclin A plasmid was cut with BamHI and transcribed with SP6 RNA polymerase, the cyclin B plasmid was cut with HindIII and transcribed with SP6 RNA polymerase, the cyclin D1 plasmid was cut with BamHI and transcribed with T7 RNA polymerase, the cyclin D2 plasmid was cut with XhoI and transcribed with T3 RNA polymerase, the cyclin D3 plasmid was cut with BamHI and transcribed with T7 RNA polymerase, the cyclin E plasmid was cut with BamHI and transcribed with T7 RNA polymerase.

The p19 fusion protein was then used in an *in vitro* association assay with 35S methionine labelled proteins from *in vitro* transcription/ translation of CDC2, CDK2, CDK4 and the various cyclins.

Ten mg of glutathione-S-transferase (GST) or GST-p19 fusion proteins bound to glutathione agarose beads as incubated with *in vitro* translated 35S-labeled cyclins or CDKs for 2 hours at 40C in 200 ml of binding buffer (20 mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 0.05% NP-40). The beads were first washed 4 times with the above binding buffer. They were then boiled and resolved on a 10% polyacrylamide gel.

GST protein alone had no affinity for CDK4, but GST-p19 protein associated with the *in vitro* labelled CDK4 protein. The GST-p19 protein does not associate with either CDC2, CDK2, cyclin A, cyclin B, cyclin D1, cyclin D2, cyclin D3 or cyclin E. GST-p19 associates specifically with the G1 cyclin dependent kinases, a property exhibited by the p16/p15 but not p21/p27 cell cycle inhibitors.

Example 4: p19 associates with CDK4/CDK6 in vivo

To examine the activity of p19 *in vivo*, we first generated rabbit antisera for the mouse p19 for analysis of p19 *in vivo*. To generate polyclonal antibodies for mouse p19, fusion protein was injected into New Zealand rabbits at 2 week intervals. Rabbits were terminally bled after 5 injections. The resulting antisera were then tested for their ability to precipitate *in vitro* translated mouse p19. Antisera were then pre-cleared on a GST affinity column and purified on a GST-p19 affinity column. Affinity resins were made by conjugation of the corresponding bacterial fusion protein to the A20 gel matrix (Biorad) per manufacturer's instruction. Antibodies for CDK4 epitope (amino acids 282-303) and CDK6 epitope (amino acids 306-326) were purchased from Santa Cruz Inc.

The purified antibodies were then used in immunoprecipitation experiments. DO11.10 cells were stimulated with 10 ng/ml PMA (phorbol 13-myristate 12-acetate) plus 0.5 mM ionomycin (Sigma) for various time points. Cells were labeled with ^{35}S methionine Translabel (ICN) in RPMI medium without methionine supplemented with 10% dialyzed fetal calf serum for 3 hours. Immunoprecipitations were performed as described (Xiong *et al.* (1992) *Cell* 71: 505-514). For sequential immunoprecipitation, the immunoprecipitates were boiled in IP buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 20 mM EDTA, 0.5% NP40 and various protease inhibitors) with 2% SDS for 20 minutes. The resulting supernatant was diluted 1:25 in IP buffer and the second immunoprecipitation was performed the same way as in the first immunoprecipitation. Samples were resolved electrophoretically on 17.5% polyacrylamide gels.

To detect CDK4 and the related kinase CDK6, we also used the corresponding antibodies (Santa Cruz Inc). As DO11.10 T cell hybridoma cells are arrested at G1 phase starting at 5 hours after stimulation with a combination of phorbol ester PMA and calcium ionophore ionomycin, we examined the state of p19 at different time points after stimulation. Cells were labelled with ^{35}S methionine and stimulated with PMA and ionomycin for 0, 3 and 6 hours. Whole cell extracts were made from these cells and

immunoprecipitation was carried out using antibodies specific for CDK4, CDK6 and p19. Similar patterns of immunoprecipitated proteins were observed at all time points. Immunoprecipitation with CDK4 yielded an abundant CDK4 protein (~34kDa) that co-migrated with *in vitro* translated CDK4. Also co-precipitated was a lesser amount of a 19 kDa protein, which co-migrated with *in vitro* translated p19. Inclusion of a competing CDK4 peptide in the immunoprecipitation experiment resulted in the disappearance of the 34 and 19 kDa proteins, indicating the specificity of the antibodies.

Using anti-CDK6 antibodies, a higher level of co-precipitated 19 kDa and an additional 16 kDa proteins was observed, which again was competed away with CDK6 peptide. The 19 kDa protein again co-migrated with the *in vitro* translated p19 protein, whereas the 16 kDa protein co-migrated with *in vitro* translated p16 protein, suggesting that they correspond to the p19 and p16 cell cycle inhibitors, respectively. No p27, p21 or p15 inhibitors were evident. Using p19 specific antibody, a 19 kDa protein as well as 34 and 40 kDa proteins were observed. The bands from the 34 and 40 kDa proteins were diminished or eliminated when a competing GST-p19 protein was included in the reaction. Thus, both CDK4 and CDK6 proteins were present in molar excess relative to the level of the associating p19.

In order to ascertain the identity of the 19 kDa protein co-precipitated with the CDK6 antibodies, we performed a double immunoprecipitation experiment. We chose CDK6 because it is known to be a major G1 kinase in T cells (Meyerson and Harlow (1994) *Mol. Cell. Biol.* 14: 2077-2086). Second immunoprecipitation of the CDK6 immunocomplex with anti-p19 antibodies yielded the p19 protein. As a control, similarly purified rabbit antisera specific for the Sp3 transcription factor or the pre-immune sera were used. As expected, they did not precipitate the 19 kDa protein. In a reciprocal experiment, anti-CDK6 antibodies precipitated a 40 kD CDK6 protein from the anti-p19 immunocomplex. Thus, p19 associates with CDK6 protein *in vivo*. Similar experiments also showed that p19 associates with CDK4 *in vivo*.

Example 5: p19 inhibits cyclinD-CDK4 but not cyclinE-CDK2 kinase activity

The *in vitro* effect of p19 on the cyclin dependent kinase activity was tested in a kinase inhibition assay. 106 insect Sf9 cells were infected with either cyclin D1 or CDK4 recombinant baculoviruses alone, or coinfected with both viruses at a multiplicity of infection of 5. Cyclin E and CDK2 recombinant baculoviruses were also used to coinfect Sf9 cells. After 72 hours, cells were resuspended in 500 ml kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl, 1 mM dithiothreitol, 0.5 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM β-glycerophosphate) plus 1 mg/ml antipain, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 0.1 mM Phenyl methyl sulfonyl fluoride (PMSF) and lysed by passing the cells through a 26G1/2 needle 6 times. The cleared lysates were aliquoted and saved at -80°C. For the kinase assays, 2 ml of insect cell lysates were mixed with various amount of bacterially expressed glutathione-S-transferase (GST), or GST-p19, or GST-p16 in 50 ml of kinase buffer and pre-incubated at 30°C for 30 to 40 minutes (Xiong *et al.* (1993) *Nature* 366: 701-704). This mixture was then added to the bacterially expressed GST-Rb large pocket protein (0.5 mg) immobilized on glutathione agarose beads plus 50 mCi of [32P-g] ATP in 50 ml of kinase buffer, and incubated at 30°C for 10 min. The phosphorylated proteins were separated with 10% SDS/PAGE, and exposed to X-ray film.

As controls, Sf9 cells infected with a combination of cyclin E and CDK2 baculoviruses or from cells infected with either cyclin D or CDK4 alone were used. GST-Rb fusion protein was used as a kinase substrate. As expected, only co-infection of cyclinD and CDK4 baculoviruses resulted in extract with kinase activity. Addition of increasing amount of GST-p19 fusion protein (but not GST alone) led to inhibition of the cyclinD/CDK4 kinase activity. Inhibition was not observed when an equivalent amount of GST-p19 was added to the kinase reaction mediated by cyclinE/CDK2. These data are consistent with the finding presented herein that p19 associates with CDK4 but not with CDK2.

For comparison, GST-p16 was used to inhibit the kinase activity of cyclin D/CDK4. The results showed that p19 is similar to p16 in its ability to inhibit the kinase activity of CDK4 but not that of CDK2.

Example 6: Activation of DO11.10 T cell hybridoma by PMA/Ionomycin arrests cells at G1.

DO11.10 T cell hybridoma cells were stimulated with 10ng/ml of PMA and .5 μM ionomycin and an aliquot of the culture was analyzed for its cell cycle profile by propidium iodide staining at different times. Cells were harvested and washed twice with PBS. The cell pellet was resuspended in 0.1 ml PBS and fixed with 0.9 ml of methanol at -70°C. Cells were washed twice in PBS, and resuspended in 0.5 ml of RNase A (2 mg/ml) and 0.5 ml of propidium iodide (20 mg/ml). Samples were analyzed by flowcytometry using the Coulter EPICS XL machine. The percentage of live cells at various stages of the cell cycle were determined by a multicycle analysis program. The results showed that PMA/ionomycin arrests T cell hybridoma cells at G1.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

All publications and patent applications cited in this specification are herein incorporated by reference in their entirety for all purposes as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: p19: A Cell Cycle Inhibitor

(iii) NUMBER OF SEQUENCES: 6

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(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94105-1492

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT Not yet assigned
(B) FILING DATE: Not yet assigned
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/425,093
(B) FILING DATE: 17-APR-1995

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 706 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..498
(D) OTHER INFORMATION: /product= "human p19"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 166 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Met Phe Gly Ser Thr Ala Ile Ala Leu Glu Leu Leu Lys Gln Gly
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65                  70                  75                  80

Ala Ala Arg Thr Gly Phe Leu Asp Thr Leu Lys Val Leu Val Glu His
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Gly Ala Asp Val Asn Val Pro Asp Gly Thr Gly Ala Leu Pro Ile His
            100                 105                 110

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1010 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 28..525
(D) OTHER INFORMATION: /product= "murine p19"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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175                 180                 185                 190

CGC CGC CTT CTT CAC CGG GAG CTG GTG CAT CCT GAC GCC CTG AAC CGC    147
Arg Arg Leu Leu His Arg Glu Leu Val His Pro Asp Ala Leu Asn Arg
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GCT TTG GAG CTC CTG AAG CAA GGT GCC AGC CCC AAT GTC CAA GAT GCC        243
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        225                 230                 235

TCC GGT ACT AGT CCT GTG CAT GAT GCG GCT CGC ACC GGG TTC CTG GAC        291
Ser Gly Thr Ser Pro Val His Asp Ala Ala Arg Thr Gly Phe Leu Asp
    240                 245                 250

ACC CTG AAG GTT CTG GTG GAG CAT GGT GCT GAT GTC AAT GCC CTG GAC        339
Thr Leu Lys Val Leu Val Glu His Gly Ala Asp Val Asn Ala Leu Asp
255                 260                 265                 270

AGC ACT GGG TCG CTC CCC ATC CAT CTG GCG ATA AGA GAG GGC CAT AGC        387
Ser Thr Gly Ser Leu Pro Ile His Leu Ala Ile Arg Glu Gly His Ser
                275                 280                 285

TCC GTG GTC AGC TTC CTA GCT CCT GAA TCT GAT CTC CAC CAC AGG GAC        435
Ser Val Val Ser Phe Leu Ala Pro Glu Ser Asp Leu His His Arg Asp
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TGGTGTTGAT TTCTTGAGTG TGTGTGTTTG GGGGGTGTTT CTCATTTGTT TTTCTCACCC      705

CTTTTGGTGT GTTGGACAAA GAAGGGGCTC CTACAGGCAA CAGCATCTAA ACGGTTCAGT      765

TTCCTCTGCA CTGGGGCTGC ACCAGGGCAG GGGTTTAAAG CCCTAGCCTC AGAGTGAGGT      825

CATCACTTCC CGGGCCCCTT GGAAGCTGGT GACCTTGGCA GGCTGTGCTC AGAGAGCCCT      885

GAAGTGTGAG CCATCTACTT TGGGCATGTG AGGGGGAAGG GAAAGCATTT CAAATCAATT      945

AAAAGGATAA CATGAGTTCA TTTTTCTTTC TTGGAAGGTT TCCAGTCTGT TGTACAGAGT     1005

TTGAA                                                                 1010
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 166 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Leu Leu Glu Glu Val Cys Val Gly Asp Arg Leu Ser Gly Ala Arg
 1               5                  10                  15

Pro Arg Gly Asp Val Gln Glu Val Arg Arg Leu Leu His Arg Glu Leu
            20                  25                  30

Val His Pro Asp Ala Leu Asn Arg Phe Gly Lys Thr Ala Leu Gln Val
        35                  40                  45

Met Met Phe Gly Ser Pro Ala Val Ala Leu Glu Leu Leu Lys Gln Gly
    50                  55                  60

Ala Ser Pro Asn Val Gln Asp Ala Ser Gly Thr Ser Pro Val His Asp
65                  70                  75                  80

Ala Ala Arg Thr Gly Phe Leu Asp Thr Leu Lys Val Leu Val Glu His
                85                  90                  95

Gly Ala Asp Val Asn Ala Leu Asp Ser Thr Gly Ser Leu Pro Ile His
            100                 105                 110

Leu Ala Ile Arg Glu Gly His Ser Ser Val Val Ser Phe Leu Ala Pro
        115                 120                 125

Glu Ser Asp Leu His His Arg Asp Ala Ser Gly Leu Thr Pro Leu Glu
    130                 135                 140

Leu Ala Arg Gln Arg Gly Ala Gln Asn Leu Met Asp Ile Leu Gln Gly
145                 150                 155                 160

His Met Met Ile Pro Met
                165
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..157
(D) OTHER INFORMATION: /note= "human p16"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Asp Pro Ala Ala Gly Ser Ser Met Glu Pro Ser Ala Asp Trp Leu
1               5                   10                  15

Ala Thr Ala Ala Ala Arg Gly Arg Val Glu Glu Val Arg Ala Leu Leu
            20                  25                  30

Glu Ala Val Ala Leu Pro Asn Ala Pro Asn Ser Tyr Gly Arg Arg Pro
        35                  40                  45

Ile Gln Val Met Met Met Gly Ser Ala Arg Val Ala Glu Leu Leu Leu
    50                  55                  60
```

```
Leu Leu His Gly Ala Glu Pro Asn Cys Ala Asp Pro Ala Thr Leu Thr
65                  70                  75                  80

Arg Pro Val His Asp Ala Ala Arg Glu Gly Phe Leu Asp Thr Leu Val
                85                  90                  95

Val Leu His Arg Ala Gly Ala Arg Leu Asp Val Arg Asp Ala Trp Gly
            100                 105                 110

Arg Leu Pro Val Asp Leu Ala Glu Glu Leu Gly His Arg Asp Val Ala
        115                 120                 125

Arg Tyr Leu Arg Ala Ala Ala Gly Gly Thr Arg Gly Ser Asn His Ala
    130                 135                 140

Arg Ile Asp Ala Ala Glu Gly Pro Ser Asp Ile Pro Asp
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 136 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..136
(D) OTHER INFORMATION: /note= "human p15"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Arg Glu Glu Asn Lys Gly Met Pro Ser Gly Gly Gly Ser Asp Glu
1               5                   10                  15

Leu Ala Thr Pro Ala Arg Gly Leu Val Glu Lys Val Arg His Ser Trp
            20                  25                  30

Glu Ala Gly Ala Asp Pro Asn Gly Val Asn Arg Phe Gly Arg Arg Ala
        35                  40                  45

Ile Gln Val Met Met Met Gly Ser Ala Arg Val Ala Glu Leu Leu Leu
    50                  55                  60

Leu His Gly Ala Glu Pro Asn Cys Ala Asp Pro Ala Thr Leu Thr Arg
65                  70                  75                  80

Pro Val His Asp Ala Ala Arg Glu Gly Phe Leu Asp Thr Leu Val Val
                85                  90                  95

Leu His Arg Ala Gly Ala Arg Leu Asp Val Arg Asp Ala Trp Gly Arg
            100                 105                 110

Leu Pro Val Asp Leu Ala Glu Glu Arg Gly His Arg Asp Val Ala Gly
        115                 120                 125

Tyr Leu Arg Thr Ala Thr Gly Asp
    130                 135
```

WHAT IS CLAIMED IS:

1. An isolated polypeptide comprising at least 10 contiguous amino acids from a polypeptide sequence selected from the group consisting essentially of SEQ ID NO:2 and SEQ ID NO:4, wherein:

said polypeptide, when presented as an immunogen, elicits the production of an antibody which specifically binds to a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4; and

said polypeptide does not bind to antisera raised against a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4 which has been fully immunosorbed with a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO4.

2. The isolated polypeptide of claim 1 selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

3. The isolated polypeptide of claim 1, wherein said polypeptide inhibits the kinase activity of cyclin D1/CDK4, and wherein said polypeptide does not inhibit the kinase activity of cyclin E/CDK2.

4. The isolated polypeptide of claim 1, wherein the polypeptide is about 19 kDa.

5. The isolated polypeptide of claim 1, wherein the polypeptide binds to CDK4 *in vitro*.

6. The isolated polypeptide of claim 1, wherein the polypeptide is recombinantly produced.

7. An immunogenic composition comprising the polypeptide of claim 1.

8. The immunogenic composition of claim 7, wherein the polypeptide of claim 1 is covalently linked to a second polypeptide.

9. An isolated nucleic acid encoding a polypeptide comprising at least 10 contiguous amino acids from a polypeptide sequence selected from the group consisting essentially of SEQ ID NO:2 and SEQ ID NO:4, wherein:

said polypeptide, when presented as an immunogen, elicits the production of an antibody which specifically binds to a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4; and

said polypeptide does not bind to antisera raised against a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4 which has been fully immunosorbed with a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

10. The nucleic acid of claim 9, wherein said nucleic acid hybridizes to a clone of the human p19 gene present in a human genomic library under stringent conditions.

11. The nucleic acid of claim 9, wherein said nucleic acid hybridizes to a clone of the mouse p19 gene present in a human genomic library under stringent conditions.

12. The nucleic acid of claim 9 selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

13. The nucleic acid of claim 9, wherein the nucleic acid further comprises a recombinant vector.

14. The nucleic acid of claim 9, wherein the nucleic acid further comprises an expression vector.

15. An antibody which specifically binds a polypeptide comprising at least 10 contiguous amino acids from a polypeptide sequence selected from the group consisting essentially of SEQ ID NO:2 and SEQ ID NO:4, wherein:

said polypeptide, when presented as an immunogen, elicits the production of an antibody which specifically binds to a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4; and

said polypeptide does not bind to antisera raised against a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4 which has been fully immunosorbed with a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

16. The antibody of claim 15, wherein the antibody is monoclonal.

17. A recombinant cell comprising a nucleic acid encoding a polypeptide comprising at least 10 contiguous amino acids from a polypeptide sequence selected from the group consisting essentially of SEQ ID NO:2 and SEQ ID NO:4, wherein:

said polypeptide, when presented as an immunogen, elicits the production of an antibody which specifically binds to a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4; and

said polypeptide does not bind to antisera raised against a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4 which has been fully immunosorbed with a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

18. The recombinant cell of claim 17, wherein said recombinant cell is prokaryotic.

19. A cell comprising an antibody which binds a polypeptide comprising at least 10 contiguous amino acids from a polypeptide sequence

selected from the group consisting essentially of SEQ ID NO:2 and SEQ ID NO:4, wherein:

said polypeptide, when presented as an immunogen, elicits the production of an antibody which specifically binds to a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4; and

said polypeptide does not bind to antisera raised against a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4 which has been fully immunosorbed with a polypeptide selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

```
                                 20                  40                60
                                  |                   |                 |
HUMAN p19         MLLEEVRAGDRLSGAAARGDV QEVRRLLHRELVHPDALNRF GKTALQVMMFGSTAIALELL
MOUSE p19         ......CV.DR....RP.... .................... ............P.V.....
HUMAN p16     MDPAAGSSM.PS.DW-.AT.....R. E...A..EAVAL-.N.P.SY .RRPI....M..ARV.EL..
HUMAN p15     MREENKGMPSGGGSDE.AT-P...L- V.KV.HSWEAGAD.NGV... .RR.I....M..ARV.EL..
                       _________1ST ANKYRIN______________ ___________2ND ANKYRIN

                                 80                  100               120
                                  |                   |                 |
HUMAN p19         KQGASPNVQDTSG-TSPVHDA ARTGFLDTLKVLVEHGADVN VPDGTGALPIHLAVQEGHTA
MOUSE p19         ..........A..-....... .................... AL.S..S......IR...SS
HUMAN p16         LH..E..CA.PATL.R..... ..E......V..HRA..RLD .R.AW.R..VD..EEL..RD
HUMAN p15         LH..E..CA.PATL.R..... ..E......V..HRA..RLD .R.AW.R..VD..EER..RD
                  __________ ___________3RD ANKYRIN_________ _____________4TH

                                 140                 160
                                  |                   |
HUMAN p19         VVSFLAAESDLHRRDARGLT PLELALQRGAQDLVDILPGH MVAPL
MOUSE p19         ......P.....H...S... ......R.....N.M...Q.H MMIPM
HUMAN p16         .ARY.R.AAGGT.GSNHARI DAAEGPSDIPD
HUMAN p15         .AGY.RTATGD
                  ANKYRIN__________
```

FIG. 1.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05252

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL : 530/350, 387.9; 514/2; 435/69.1, 240, 252.3, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 387.9; 514/2; 435/69.1, 240, 252.3, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	Hirai et al. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Molecular and Cellular Biology. May 1995, Vol. 15, No. 5, pp.2672-2681, especially 2673-2678.	1-14,17,18
A	Hannon et al. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature. September 1994, Vol. 371, pp. 257-261.	1-14,17,18
A	Serrano et al. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature. December 1993, Vol. 366, pages 704-707.	1-14,17,18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 21 MAY 1996	Date of mailing of the international search report 15 JUL 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer KENNETH A. SORENSEN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05252

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	Yeudall et al. Cyclin kinase inhibitors add a new dimension to cell cycle control. Oral Oncol, Eur J Cancer. May 1995, Vol 31B, No5. pp. 291-298.	1-14,17,18
X,P	Ming et al. Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16ink4. Molecular and Cellular Biology. May 1995, Vol. 15, No. 5, pp. 2682-2688, especially 2683-2687.	1-14,17,18
Y,P	Mao et al. A Novel p16ink4A transcript. Cancer Research. 15 July 1995, Vol. 55, pp. 2995-2997, especially, page 2995-2996.	1-14,17,18
Y,P	Stone et al. Complex structure and regulation of the P16 (MTS1) locus. Cancer Research. 15 July 1995, Vol. 55, pages 2988-2994.	1-14,17,18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05252

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-14, 17-18

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05252

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/00, 39/00; C07H 21/04; C07K 1/00, 16/00; A01N 37/18; C12P 21/00; C12N 5/00, 1/20, 15/00;

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN: MEDLINE, BIOSIS, EMBASE, CONFSCI, DISSABS, PATOSEP, WPIDS, JICST-EPLUS
search terms: cell cycle inhibitor, P19 and synonyms, cyclin D1/CDK4, antibody, cDNA, recombinant, protein, amino acid sequence

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-14, 17-18 drawn to an isolated polypeptide, and a nucleic acid encoding a recombinant form of said polypeptide.
Group II, claim(s) 15-16, 19, drawn to an antibody.
The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Group I consists of claims drawn to a polypeptide, a nucleic acid encoding the polypeptide, an expression vector, a transformed cell, and an immunogenic composition of the polypeptide. The shared special technical feature is the nucleic acid or amino acid sequence encoding the claimed polypeptide.
Group II consists of claims drawn to a antibodies that specifically bind the claimed polypeptide, and as such do not share the special technical feature of a sequence found in the polypeptide claimed for Group I.

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